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Effects of 0.1 ppm Nitrogen Dioxide on Airways of Normal and Asthmatic Subjects

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16. ABSTRACT

It has been reported (J. Clin. Invest. 57: 301-307, 1976) that inhalation of nitrogen dioxide (NO_2) will enhance the bronchial reactivity of asthmatics. This study was designed to evaluate the respiratory effect of a 1-h exposure of normal subjects and of atopic asthmatics to 0.1 parts per million (ppm) NO_2. Fifteen normal and 15 asthmatic subjects were exposed to air and to NO_2 in randomized double-blind crossover design. Exposure to either atmosphere was bracketed by bronchial inhalation challenge using aerosolized metacholine chloride solutions. Plethysmographic measurements of specific airway resistance (sRaw) and the forced random noise impedance spectrum (5-30 Hz) were obtained immediately after each methacholine dose. Following acute exposure to NO_2, there was a slight but not significant increase in mean base-line resistive properties of the respiratory system determined by forced random noise excitation were not significantly affected by NO_2 inhalation either. Finally, there was no change in bronchial response to methacholine challenge in either group. These findings indicate that 0.1 ppm NO_2 exposure for 1 h without exercise had no demonstrable airways effects in either young atopic asthmatics with mild disease or young normal subjects.

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Effects of 0.1 ppm nitrogen dioxide on airways of normal and asthmatic subjects

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atopic asthmatics; airway resistance; bronchial challenge test; methacholine chloride; forced random noise

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RECENT REPORTS by European investigators (20, 21) have raised the concern that short-term exposures to nitrogen dioxide (NO_2) in the range of 0.1–0.2 parts per million (ppm) may adversely affect pulmonary function in asthmatic subjects. These authors used a bronchoconstrictor challenge to elicit more subtle effects than might be evident using more traditional measurements of pulmonary performance. Earlier studies of short-term exposure (1–2 h) (8, 12, 16) employing traditional measures of pulmonary function in healthy subjects did not disclose any significant effects even at NO_2 levels up to 1.5 ppm. However, following exposure of asthmatic subjects for 1 h to 0.1 ppm NO_2, Orehek et al. (21) reported statistically significant increases both in specific airway resistance and in bronchial response to inhalation of a carbachol aerosol. The authors suggested that asthmatic subjects or other similarly sensitive subgroups might be "at risk" for adverse effects on pulmonary performance at NO_2 levels well below those previously found to be innocuous in healthy subjects.

Chronically increased bronchial reactivity to inhaled aerosolized bronchoconstrictor drugs is a feature of spontaneous asthma and may also occur in occupation-related asthma (13). Transient increases in bronchial reactivity have been described following viral respiratory tract infections and short exposures to ozone even after postexposure lung mechanics have returned to normal (3). Excessive bronchial reactivity has been suggested to constitute a risk factor in the development of chronic obstructive lung disease (COLD) (5). Thus bronchial provocation testing with nonspecific bronchoconstrictor drugs may be a sensitive test for detection of significant airways abnormalities.

The purpose of the present study was to assess the findings of Orehek et al. (21) by conducting a similar controlled clinical exposure of an asthmatic group to 0.1 ppm NO_2 using a bronchial challenge technique in addition to the more traditional assessment of pulmonary function.

METHODS

Subjects. Two groups of male subjects, normals and atopic asthmatics, were selected for this study. These nonsmoking volunteers between the ages of 18 and 35 yr underwent comprehensive screening that included *1*) medical history (Duke University computerized history form), *2*) Spielberger State/Trait Anxiety Inventory (STAI) answered before entry into the study (Trait) and before and after each bronchial challenge (State), *3*) physical examination, *4*) blood chemistry screen (SMA 20), *5*) complete blood count (CBC) with differential, *6*) a series of 16 epicutaneous scratch skin tests performed with 0.01 ml of 1:20 wt/vol antigen extracts characteristic for the North Carolina region (Greer Laboratories, Lenoir, NC), *7*) epicutaneous skin tests with four concentrations of methacholine chloride (Mecholyl Chloride), *8*)

spirometry, and *9*) bronchial inhalation challenge with methacholine (4, 6). To be admitted into the normal group, the subject had to meet the following criteria: *1*) no history of asthma, hay fever, or atopic dermatitis, *2*) no occupational exposure to dusty or caustic environment, *3*) no smoking history within 6 mo, *4*) no 3-plus (≥12 mm wheal) or 4-plus (≥12 mm wheal with pseudopod formation) scratch skin test, *5*) forced expired volume at 1s-to-forced vital capacity ratio (FEV_1/FVC) ≥ 75% and FVC ≥ 80% of predicted normal value (17), *6*) airway resistance (Raw) ≤ 2.0 $cmH_2O \cdot l^{-1} \cdot s$ (body plethysmography), and *7*) less than 100% increase in Raw (above base-line Raw) at 5 mg/ml concentration of methacholine during a screening bronchial challenge test (see below).

Subjects classified as asthmatics had mild disease and had to satisfy the following conditions: *1*) positive past history of reversible wheezing and dyspnea not associated with respiratory infection, *2*) no exposure to dusty or caustic environment, *3*) $FEV_1/FVC \geq 70\%$ and FVC ≥ 70% of predicted or 80% of the patient's best recorded value in the past year, *4*) Raw ≤ 3.0 $cmH_2O \cdot l^{-1} \cdot s$, *5*) at least 100% increase in Raw above base line in response to inhalation of methacholine aerosol at 2.5 mg/ml, *6*) no current steroid therapy, *7*) no daily bronchodilator therapy for the previous month, and *8*) <50 days of dyspnea per year, whether at rest or exercise. All accepted asthmatics were considered to have mild or inactive disease. None of the patients with positive aeroallergen history were excluded from the study, conducted between November and April. All subjects were asked to withhold all medications for 48 h prior to the experiment, including bronchodilators, antihistamines, and vitamins. They were also asked to abstain from all coffee, tea, colas, alcoholic beverages, and chocolate for 12 h prior to the experiment. We comprehensively screened 46 volunteers qualified for the study; 24 were classified as normals and 22 as asthmatics. Of these, 15 normals and 15 asthmatics completed the study. Most of the 16 subjects who did not complete the study withdrew for various reasons before beginning the exposure protocol. Physical characteristics of the subjects are shown in Table 1.

Standardized bronchial challenge test (SBCT). The technique used is similar to that recommended by the Committee of the Asthma and Allergic Disease Centers of the National Institute of Allergy and Infectious Diseases (NIAID) (4) and the American Thoracic Society (ATS) (6). Stock solutions of methacholine in normal saline (5 and 20 mg/ml) were prepared weekly by the hospital pharmacy, passed through a 0.22-μm Millipore filter, and stored at 4°C. Methacholine solutions for inhalation were prepared daily by diluting stock solution with sterile 0.9% NaCl. The aerosol was generated by an ultrasonic nebulizer (DeVilbiss, model 65). To narrow particle-size distribution, the nebulizer output was serially connected with a simple, laboratory-made, single-stage cascade impactor with a bias flow of 5.0 $l \cdot s^{-1}$. With the output of the nebulizer set at 1.5 ml/min, the mass median diameter of particles, depending on the concentration of methacholine, ranged between 1.7 ± 1.4 (geometric standard deviation) and 2.4 ± 1.6 μm as characterized by an electronic aerosol analyzer (TSI, model 3030) and Climet optical particle counter (model 208).

TABLE 1. *Averaged physical characteristics data for two groups of subjects*

	Age, yr	Ht, cm	Wt, kg	FVC, liters	FVC,* %	FEV_1, liters	FEV_1,* %
				Normals			
Median	27.1	181.6	67.9	5.06	91.7	4.32	92.7
Mean	27.4	182.2	70.4	5.26	91.9	4.37	90.9
±SD	±4.1	±4.3	±9.5	±0.79	±14.0	±0.72	±15.0
Min	22.9	174.0	58.3	4.23	67.3	3.27	66.6
Max	39.1	190.0	91.7	6.66	115.0	5.62	109.2
PCS	0.22	0.40	0.78	0.76	0.04	0.21	−0.36
				Asthmatics			
Median	26.4	178.0	72.2	4.53	83.0	3.55	78.0
Mean	29.0	178.2	74.4	4.77	85.0	3.58	77.0
±SD	±6.9	±7.2	±8.4	±0.65	±15.0	±0.66	±17.0
Min	21.3	168.9	61.4	4.06	66.6	2.22	45.6
Max	46.1	192.0	91.6	6.13	114.2	4.39	104.4
PCS	1.13	0.08	0.79	1.12	0.40	0.12	−0.18

n = 15. FVC, forced vital capacity; FEV_1, forced expiratory volume at 1 s; SD, standard deviation; Min, minimum value of the sample; Max, maximum value of the sample; PCS, Pearsonian coefficient of skewness. * Variable expressed as percent of predicted value (27).

The actual SBCT procedure started with inhalation of five tidal breaths of 0.9% saline, followed 5 min later by another five breaths of saline. The saline challenges were followed at 5-min intervals by similar inhalation of progressively increasing concentrations of aerosolized methacholine. The methacholine aerosol used to challenge asthmatics was generated from 0.075, 0.15, 0.31, 0.62, 1.25, 2.5, and 5.0 mg/ml solutions. The healthy subjects were challenged with 0.31, 0.62, 1.25, 2.5, 5.0, 10.0, and 20.0 mg/ml solutions. The tidal volume of approximately 900 ml (750–1,100 ml) was inhaled at flow rates less than 1 $l \cdot s^{-1}$. At the end of each inspiration, the subject held his breath for 4 s. The tidal as well as total volume of inhaled aerosol was monitored with a Bourns ventilation monitor. This ventilatory mode of delivery was intended to facilitate central distribution of the aerosol (29) and to ensure larger percentage of particle retention (24). Subjects were requested not to take deep breaths, cough, or sigh during the SBCT, because it has been shown that a deep lung inflation may have a considerable effect on airway resistance (9, 23). Filter samples taken at the mouthpiece showed that the amount of inhaled methacholine measured as a dry salt was approximately 0.38 μg/l air at the lowest (0.075 mg/ml) concentration. Individual as well as cumulative doses of the methacholine in solutions [as recommended by NIAID (4) and ATS (6)] and the corresponding methacholine (dry salt) quantities delivered at the mouthpiece are given in Table 2.

Each series of five inhalations was followed 1 min later by 1–2 Raw and respiratory system impedance measurements by plethysmographic and forced random noise excitation techniques, respectively. The great majority of subjects were (during a bronchial challenge test) tested with a full range of methacholine solutions. However, in a small number of cases, the test was terminated when either the subject requested it or the Raw value reached 15 $cmH_2O \cdot l^{-1} \cdot s$.

Pulmonary function tests. The basic set of pulmonary

TABLE 2. *Solution and filter concentrations and dose units of methacholine chloride*

	Concentration/Dose									
Methacholine,* per solution, mg/ml	0.0	0.075	0.15	0.31	0.62	1.25	2.5	5.0	10.0	20.0
Methacholine,† cumulative dose, mg	0.0	0.375	1.13	2.69	5.79	12.0	24.5	49.5	99.5	199.5
As dry salt, μg/l aerosol										
Methacholine and NaCl‡	47.7	44.0		49.2		45.6		78.8		144.8
Methacholine and NaCl§	47.7	48.1	48.5	49.3	50.8	54.0	60.2	72.7	97.7	147.7
Methacholine§	0.0	0.38	0.75	1.56	3.1	6.3	12.5	25.0	50.0	100.0
Methocholine,† inhaled cumulative dose, μg	0.0	1.90	5.65	13.45	29.00	60.0	122.5	247.5	497.5	997.5

* Dissolved in nonbuffered sterile 0.9% NaCl. † Five breaths at each concentration (for details see METHODS and the NIAID (4) and ATS (6) guidelines). ‡ Obtained from paper filter samples taken at the mouthpiece (for details see METHODS). § Amounts calculated by solving the linear least-squares fit equation derived from measured (on filter‡) values.

function tests was comprised of body plethysmography, forced oscillation measurements, and spirometry. A standard variable-pressure body plethysmograph (CPI, model 2000) interfaced with a PDP 11/40 computer was used to obtain on-line values of functional residual capacity, Raw, and thoracic gas volume (TGV). Also, specific airway resistance (sRaw) was calculated as Raw × TGV. During the acquisition phase of the maneuver, subjects were aided by oscillographic display and recorded breath sounds to maintain panting frequency at 1.5 Hz with peak flows at $\pm 1\ l \cdot s^{-1}$. For actual calculation of the flow-box pressure slope, however, only points between ±0.4 and $0.5\ l \cdot s^{-1}$ of flow were used (18). Two to six breaths were averaged for calculation of the flow-box pressure slope as well as the slope of box pressure-mouth pressure with the shutter closed. In addition to the plethysmographic method, respiratory resistance was measured by the forced oscillation technique (25, 33). Briefly, subjects breathed tidally into the forced oscillation apparatus for 45 s while both random flow and pressure signals were recorded on magnetic tape for later analysis. The off-line taped analog signal was digitized at 512 Hz; the data were reduced and analyzed using spectral analysis techniques (33) to obtain the impedance spectrum. Thevenin (effective) resistances were computed over the following frequency ranges: from 5 to 9 Hz (R_{5-9}) and from 20 to 24 Hz (R_{20-24}). The ratio of low to high frequency resistance ($RR = R_{5-9}/R_{20-24}$) also was calculated. In addition, the resistance at 1 Hz (R_1) was obtained by extrapolation. The basic set of spirometric parameters was derived from a FVC maneuver performed on a dry-seal spirometer (CPI, model 220) and interfaced with a PDP 11/40 for the on-line analysis of data. Each subject participated in at least one plethysmographic training session prior to the screening session.

Immunologic and morphologic studies. These included *1*) nasal biopsies and washes obtained at times shown in Table 3 to assess changes in the ultrastructure of respiratory epithelium induced by NO_2, *2*) atraumatic biopsy of the epithelium from the inferior surface of one middle turbinate using a thin-wire loop (2), and *3*) nasal washing performed by instilling 5 ml of normal saline into one nostril, retaining the fluid for 10 s, and expelling it into a receptacle for analysis. These data will be the subject of a separate report.

Experimental protocol. After satisfying all screening requirements, each eligible subject was asked to participate within 1 wk of screening for 3 consecutive days in each of 2 consecutive wk. All subjects who accepted were randomly assigned to one of two exposure sequences: *1*) air-NO_2; *2*) NO_2-air. Both air and NO_2 exposures (188 μg/m^3; 0.1 ppm) were of 1-h duration. The two exposures for each subject were carried out in double-blind fashion on the same days of the week and the same hour of the day. On the exposure days, the time lapse between the end of exposure and the initiation of SBCT was approximately 20 min. All the tests were always performed on the same days of the week and the same hour of the day. A typical 2-wk protocol is schematically presented in Table 3. During the exposure in the chamber the subjects rested quietly most of the time. Before and after each exposure blood pressure and pulse were recorded and a symptom questionnaire was filled out by the subject. Electrocardiogram was continuously monitored via telemetry.

TABLE 3. *Experimental protocol, test sequence, and frequency of measurements*

Screening Day	*Weeks 1 and 2*		
	Day 1	*Day 2*	*Day 3*
1) PFT Battery *2*) SBCT	*1*) NB & NW *2*) PFT *3*) SBCT *4*) NB & NW	*1*) PFT *2*) 1-h exposure *3*) NB & NW *4*) PFT *5*) SBCT	*1*) PFT *2*) SBCT

NB & NW, nasal biopsy and nasal wash; PFT, pulmonary function tests: plethysmography (2 × FRC, 2 × Raw) followed by forced random noise (FRN) (2 × 75 s); SBCT, standardized bronchial challenge test: base-line (saline) (2 ×, Raw, 2 × FRN) followed by methacholine challenges (1 × Raw, 1 × FRN); FRC, functional residual capacity; Raw, airway resistance.

All exposures were performed in the stainless steel, laminar flow chambers of the Environmental Protection Agency in Chapel Hill, NC. Detailed description of the exposure facilities can be found in the EPA publication EPA-600/1-78-064 (31). Ambient conditions in the chamber, monitored throughout the protocol, were maintained at 40% relative humidity and 21°C. Mean ambient levels in ppm by volume of NO, NO_2, O_3, and SO_2 on sham days were 0.011 ± 0.009 NO, 0.006 ± 0.002 NO_2, 0.003 ± 0.001 O_3, and 0.001 ± 0.001 SO_2. During NO_2-exposure days the pollutant concentration was continuously monitored by a Bendix model 8101B NO_x analyzer and was maintained

at 0.10 ± 0.006 (SD) ppm. The total airborne particle mass was routinely <3 $\mu g/m^3$ with approximately 10^5 particles/m^3.

Analysis of methacholine dose-response data. Because the relationship between methacholine dose and subject responses is usually curvilinear, several parameters have been calculated to describe each curve: *1*) the provocative dose, *2*) the slope, and *3*) the logistic (curve-fitting) parameters.

The approach of Orehek et al. (21) was used to determine the provocation dose (PD). The PD_{100} was defined as that dose of methacholine that caused doubling of the base-line value of the sRaw. The mean base-line sRaw was calculated from pooled sRaw trials obtained on a screening day, on *day 1* of the 1st wk, and on *day 1* of the 2nd wk (see Table 3). In addition to the above method of determining PD, the dose-response curves were analyzed in terms of threshold dose, as suggested by Habib et al. (11). The threshold (PD_{2SD}) was defined as that dose of methacholine that produced an sRaw equal to mean plus two standard deviations of the pooled sRaw's obtained during base-line measurements and described in PD_{100} method.

Both approaches, however, required some special considerations, because the procedure used in this study generated only 7 dose-response curve points *vs.* 5–20 points measured by Orehek's group (21). Because the density of points, on the curve, especially in the higher methacholine concentration range was low, the PD often fell between two values (obtained during SBCT) and had to be estimated. Two techniques were evaluated on selected dose-response curves: *1*) direct calculation of PD by solving the logistic equation for the particular dose-response curve, and *2*) linear interpolation between the two nearest dose-response points. Comparison of the two methods over a range of dose-response curves showed that the absolute differences between PD values were miniscule. Consequently, we opted for the simpler linear interpolation technique in calculating all PD_{100} and PD_{2SD} values.

Another parameter used to evaluate response to methacholine challenges is the slope of the terminal portion of the dose-response curve. According to Orehek and Gayrard (22), PD reflects "sensitivity," whereas slope is a measure of the subject's "reactivity." For calculation of slopes, all observed points of the dose-response curve above plus one point below the PD (as determined by either method) were used to do a linear least-squares fit between the response and the base 10 logarithm of the cumulative methacholine salt dose following each sequential five-breath exposure. If the point below the PD was the zero dose (saline alone) value, it was not used for calculation of the slope.

To better characterize as well as maximize the amount of information from the dose-response curves, a set of 30 curves comprising a wide range of responses was selected and best fits obtained using a least-squares criterion with various functions. The functions used were power, logarithmic, exponential, quadratic, and logistic. Because the highest correlation coefficients for most of the curves were obtained by logistic fits, only this function was used for a complete data analysis. The following logistic equation was used to fit data

$$\text{response} = \frac{A}{1 + e^{-2(B + C \times \text{dose})}}$$

where response is the variable of interest, e.g., sRaw (absolute units), dose represents the cumulative dose of methacholine (mg), and *A*, *B*, and *C* are estimated parameters of the logistic curve.

Statistical analysis. Data from the four initial data sets (air-NO_2 and NO_2-air sequences for both the asthmatic and normal groups) were pooled to create only two data bases: normals and asthmatics. The principal variables of interest were sRaw and PD_{100}. In addition, the variables R_1, R_{5-9}, RR, PD_{2SD}, and slope of the dose-response curve were analyzed. Thus a total of seven variables per group were evaluated with respect to the possible effect of NO_2 exposure, with analyses done separately for normals and asthmatics. If a subject did not have complete set of data for a particular test, he was not included in the analysis. For each of the above variables, two hypotheses were tested using multivariate analysis of variance for a repeated measures type of experiment (19). The first hypothesis (H1: preair − pre-NO_2 = 0) tested the equality of the preexposure values. This hypothesis tested whether the subjects were starting the air and NO_2 exposures with the same base-line pulmonary function. The second hypothesis [H2: (preair-postair) − (pre-NO_2 − post-NO_2) = 0] tested whether the change in the means from preexposure to postexposure was the same for both the air and NO_2 exposures. This test is essentially a test for an NO_2 effect. For sRaw, R_1, R_{5-9}, and RR, the preexposure means are from the measurements made during the session conducted immediately before the beginning of exposure, whereas for the PD_{100} and slope variables, the preexposure means are from the measurements made during the SBCT done the day before the exposure (see METHODS). In addition, multivariate analysis of variance was used to test the hypothesis H3, that the response pattern of the four mean values for each parameter (pre- and postair and pre- and post-NO_2) was the same for normal and asthmatic subjects. The fourth hypothesis (H4) tested determined whether the mean of the above four means was the same for normals and asthmatics, or, in other words, whether the pulmonary function of both groups was the same. Note that H4 can be tested only if H3 is accepted. To test for a potential training effect, the hypothesis (H5) that there are no differences in the mean parameter values over the 3 air days (*day 1*, postair *day 2* and *day 3*) was tested. Although a large number of hypothesis have been tested, we declared a priori only two hypotheses H1 and H2, for the two variables sRaw and PD_{100} as of primary importance. Thus we applied a Bonferroni correction factor of 4 (2 hypotheses × 2 variables) to a *P* value of 0.05 to obtain a *P* level of 0.0125 (0.05 ÷ 4), which was used to reject null hypotheses (1).

RESULTS

Fifteen normal and fifteen atopic asthmatic subjects completed the study. The random assignment of the exposure sequence resulted in nine normals and eight

asthmatics receiving air first, whereas six normals and seven asthmatics received NO_2 first. None of the subjects detected the presence of NO_2 during these exposures. Subject symptoms, such as an occasional cough or headache, were evenly distributed between the air and NO_2 exposures.

The main variable of interest was sRaw. The averaged data for sRaw for the two groups with corresponding means ± SE are given in Table 4. Acceptance of H3 and rejection of H4 indicate that despite the mild nature of their disease ensured by the selection process at the time of study, asthmatics have higher mean base-line sRaw values. Both Raw and TGV elevations contributed to this statistically significant increase in sRaw at all four experimental conditions. However, within each group (and for each subject) the base-line sRaw among days was quite reproducible. The maximum difference between the highest and the lowest values averaged to obtain base-line sRaw for the air exposure data (solid lines) was only 6% of the average value in normals and 8% in asthmatics. R_1, R_{5-9}, and RR measured by forced oscillation technique showed similar patterns of changes (Table 4). The small changes in lung mechanics relative to base-line measures over the four consecutive testing conditions (*day 1*, pre- and postair or NO_2 of *day 2*, and *day 3*) of the normal and asthmatic groups were very similar. However, the absolute magnitude of changes for the asthmatic group was somewhat larger, because the asthmatic group had a higher mean sRaw. These between-day differences, for all the above variables, were not significant, and the hypothesis H2 could not be rejected in either group. Hypothesis H5 could not be rejected for any variable; hence, there apparently was no training effect on any variable tested. Following the NO_2 exposure mean sRaw was slightly increased in both normals (5%) and asthmatics (8%). However, neither increase in sRaw was statistically significant ($P = 0.225$ and 0.423, respectively).

TABLE 4. *Averaged resistance parameters for day 1, 2, and 3 of air- and NO_2-exposure wk*

Test	*Day 1*	*Day 2* (Air)		*Day 2* (NO_2)		*Day 3*	H1 *P* values
		Pre	Post	Pre	Post		
				Normals			
sRaw*	4.10 ±0.20	3.85 ±0.21	3.82 ±0.22	3.96 ±0.27	4.15 ±0.28	3.89 ±0.20	0.670
R_1†		1.68 ±0.13	1.76 ±0.15	1.76 ±0.19	1.81 ±0.21		0.643
R_{5-9}†		2.15 ±0.11	2.19 ±0.13	2.21 ±0.15	2.24 ±0.15		0.695
RR†		0.86 ±0.02	0.85 ±0.02	0.86 ±0.03	0.86 ±0.03		0.722
				Asthmatics			
sRaw	6.73 ±1.11	6.13 ±0.84	6.25 ±0.91	6.34 ±0.93	6.76 ±1.02	6.36 ±0.97	0.535
R_1		2.46 ±0.20	2.53 ±0.33	2.98 ±0.29	3.12 ±0.41		0.014
R_{5-9}		2.94 ±0.20	2.91 ±0.24	3.06 ±0.21	3.13 ±0.25		0.372
RR		0.90 ±0.02	0.91 ±0.02	0.97 ±0.03	0.97 ±0.04		0.009

Values are means ± SE; $n = 15$ except * $n = 14$; † $n = 11$. See text for abbreviations.

Inspection of Figs. 1 and 2, which show the individual methacholine dose-response curves of sRaw (logistic fit) for all 30 subjects following exposure to air (solid lines) and to NO_2 (broken lines), demonstrates the range of responses. The correlation coefficients for these curves were high with average *r* values for the postair and post-NO_2 dose responses in normal subjects of 0.957 and 0.969, respectively. For asthmatics, the corresponding mean *r* values were 0.967 and 0.985. On a group basis, 10 normal subjects needed a cumulative methacholine dose of 16.2 ± 2.7 (SE) mg to reach PD_{100} postair vs. 18.3 ± 3.0 mg post-NO_2. The remaining five subjects (*no. 1, 2, 4, 17*, and *20*) failed to reach PD_{100} either postair or post-NO_2, even at the maximum methacholine concentration employed and were not included in this particular analysis. The relative response of asthmatics was the same as that of normals, but the actual methacholine concentrations were smaller by almost a factor of 10. Again, two patients (*no. 56* and *62*) had to be excluded for failing to reach a postair or post-NO_2 PD_{100}. For the other 13 asthmatics, the average postair PD_{100} was 1.9 ± 0.4 mg and post-NO_2 was 2.0 ± 1.0. About half of the asthmatic patients appeared to be less sensitive to methacholine after NO_2 exposure, whereas six subjects (*no. 53, 57, 60, 62, 67*, and *69*) showed some enhancement of response to methacholine following exposure to NO_2. A similar distribution of subject responses was observed among the normal cohort. Seven subjects (*no. 1, 4, 7, 8, 14, 16*, and *20*) showed virtually no change in their methacholine dose-response curves after NO_2, and five subjects (*no. 10, 17, 19, 21*, and *24*) showed a decreased response, whereas in three subjects (*no. 2, 3*, and *12*) the response was enhanced.

To determine whether some of the subjects could be classified as "responders" to NO_2, the same criteria as those used by Orehek et al. (21) were employed. The criterion of at least a 20% fall of PD_{100} values between control (postair) and post-NO_2 conditions identified three asthmatics (*no. 53, 57*, and *69*) as responders. The averaged PD_{100} (cumulative dose) for these three asthmatic responders, post-NO_2 trial, was 1.8 mg vs. 3.4 mg for the postair challenge.

The graphical presentation of mean values for PD_{100} ± SE for sRaw is given in the left-hand panel of Fig. 3. None of the between-day differences for either group were statistically significant. However, between-group differences were significant, with asthmatics requiring much lower doses. Similar absence of response to NO_2 was observed in analyzing the PD_{100} values for the forced random noise respiratory resistance parameters, R_1, R_{5-9}, and RR.

The absolute values of PD_{2SD} and PD_{100} for sRaw differed up to 25% when derived from the same raw data. The between-days (*day 1* vs. *day 2*) within-group differences also ranged up to 22%. More importantly, however, the direction of NO_2-induced changes as measured by PD_{100} and PD_{2SD} was the same, and neither variable showed statistical significance for any hypothesis tested.

The slope of the terminal portion of the log dose-response (sRaw) curve (related to reactivity) was not significantly altered by NO_2 exposure either. The right-hand panel of Fig. 3 shows changes in mean slope ± SE

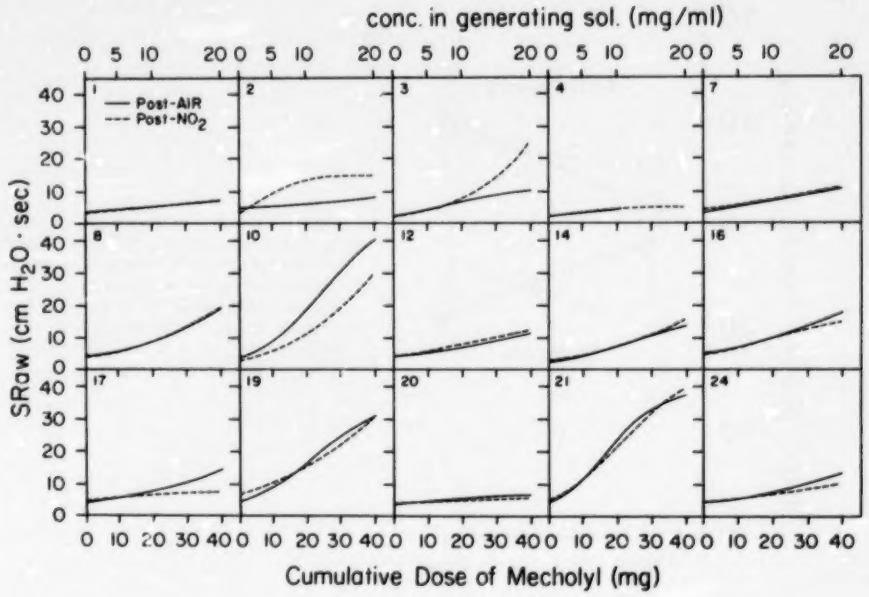

FIG. 1. Individual postair (*solid lines*) and post-NO_2 (*broken lines*) responses to bronchial challenge for 15 normals. Methacholine dose-response curves are presented in form of logistic curves.

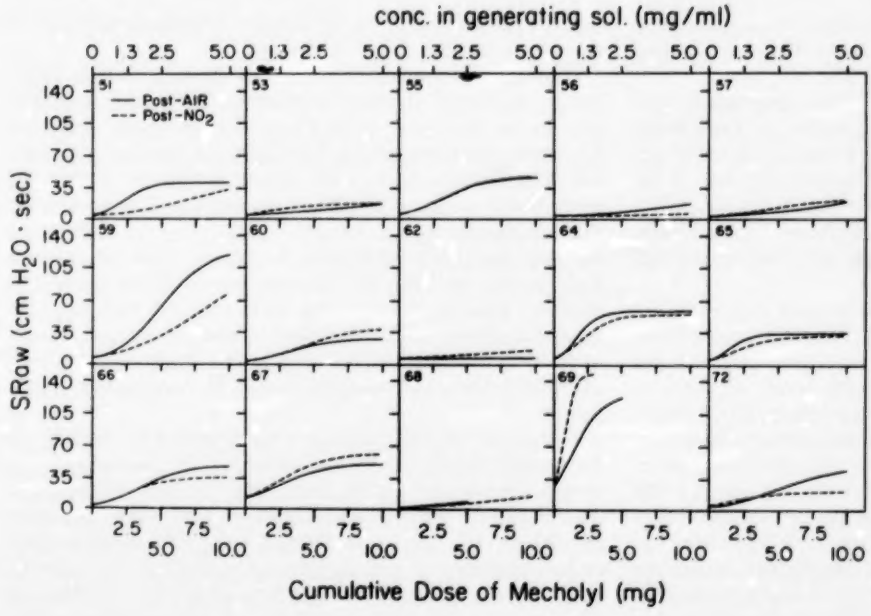

FIG. 2. Individual postair (*solid lines*) and post-NO_2 (*broken lines*) responses to bronchial challenge for 15 asthmatics. Methacholine dose-response curves are presented in form of logistic curves.

for the two groups. None of the changes in average reactivity were significant. Multivariate analysis of variance showed that neither the within-group mean differences between pre- and postair and pre- and post-NO_2 (H2 hypothesis) nor the between-group (normal vs. asthmatic; H3 hypothesis) differences were significant. A similar absence of response was observed for the log dose-response slope of the forced oscillation resistance parameters, R_1, R_{5-9}, and RR. Additional statistical analysis of reproducibility of PD_{100} and slope values for sRaw, R_1, R_{5-9}, and RR responses revealed better reproducibility for slopes than for PD_{100} in normals, whereas the inverse was true for asthmatics. Acceptance of hypothesis H5 indicates that there is no evidence of training effect for any of the four parameters tested in either group.

DISCUSSION

Our data indicate that a 1-h exposure to 0.1 ppm NO_2 caused a slight, but not significant, increase in mean sRaw in both normals and asthmatics. This finding is in agreement with the results of Orehek et al. (21) who also reported similar though statistically significant increase in mean sRaw following NO_2 exposure for a selected group of subjects. The percentage increase in averaged sRaw in these two studies (5–8%) was greater than that reported (in normal subjects) by other investigators (2–

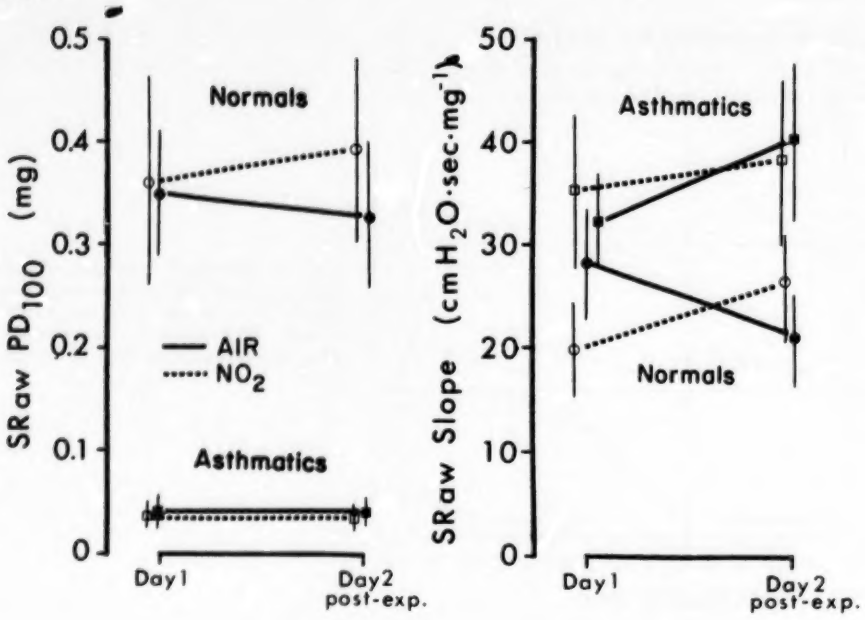

FIG. 3. Mean ± SE provocative dose (*left panel*) and mean ± SE slopes (*right panel*) over terminal portion of log dose-response curves for normals ($n = 7$; *circles*) and asthmatics ($n = 13$; *squares*) obtained before (*solid lines*) and after (*broken lines*) exposure to NO_2. *Day 1* data are taken as preexposure measures.

5%), despite the fact that in those experiments subjects were exposed for longer durations to five- to six-fold greater concentrations of NO_2 (12, 16).

The overall resistive properties of the respiratory system, measured independently using forced random noise superimposed on tidal breathing, were not acutely affected by NO_2 inhalation either, because the forced oscillation variables R_1 and R_{5-9} showed only a statistically nonsignificant 3–5% post-exposure increase. These observations are consistent with findings reported on normal or asthmatic subjects (12).

Laboratory exposures of animals as well as accidental exposures of humans to high NO_2 concentrations have shown NO_2 to be specifically injurious to small airways (27, 28). Thus already compromised small airways of asthmatics might be especially susceptible to NO_2 effects. To detect potential impairment of small airways function, we used frequency dependence of effective resistance (the RR measure), because Hayes et al. (14) showed RR to be sensitive to early airways disease in asymptomatic cigarette smokers with normal spirometry. However, no increased frequency dependence of respiratory resistance was observed. This suggests that there was no selective increase in peripheral airway resistance in asthmatics following NO_2 exposure.

Evaluation of bronchial responsiveness to methacholine showed that the asthmatic group was, as expected, much more sensitive (PD_{100}) and somewhat more reactive (slope of the log dose-response curve) to methacholine than normals. Although direct quantitative data comparison among various studies including ours is difficult to make because of different analytical approaches, our observations agree qualitatively with data reported by other investigators (7, 30). The NO_2-induced enhancement of bronchial responsiveness reported by Orehek et al. (21) was not observed, however. Although the experimental design in both studies was similar, might variation in methodology or in subject selection account for the observed difference in results?

The aerosol-generating systems, despite design differences, appeared to produce particles with approximately the same physical characteristics. Because subjects' breathing patterns during inhalation of aerosol were similar, the distribution of bronchoconstrictors within the respiratory system should be comparable. The acetylcholine analogues used as bronchoconstrictors in these studies are chemically different; however, their pharmacologic action, at least for the purpose of these studies, is similar. Both carbachol and methacholine will produce maximal effects within minutes of administration. Moreover, because of relatively prolonged duration of action of both drugs, their effects should be considered cumulative.

Although the generating and inhalation techniques were very similar, two different methods were employed to administer aerosol. In our study, the concentration of methacholine solution was doubled every 5 min (geometric series), whereas in the Orehek et al. (21) study, a more or less constant concentration of carbachol was inhaled at 2- to 4-min intervals (arithmetic series). Additional dilution of generated aerosol by air before delivery further decreased the concentration of methacholine. Direct filter sampling at a mouthpiece showed that the amount of methacholine deposited on a mouthpiece filter is only a fraction of the dose nebulized (see Table 2).

The most critical factor, however, determining the magnitude of response is the amount of inhaled drug. Because Orehek et al. (21) did not measure quantitatively the amount of carbachol inhaled (at a mouthpiece), it is more difficult to compare dosage of drugs between the two studies. In our study, the quantity of bronchoconstrictor drug available in the first inhalation dose was only 0.38 μg/l methacholine aerosol vs. 23.2 μg/l carbachol aerosol. Despite much faster accumulation rate of

methacholine, the maximum dose of the drug our asthmatics could have inhaled was 0.25 mg. This amount is equivalent to approximately 12 breaths of carbachol and is obviously much less than the maximum of 1.6 mg carbachol inhaled by some asthmatics in the Orehek (21) study. Thus it is clear that although the doses of carbachol and methacholine, respectively, were consistent within studies, the inhaled amounts of aerosolized drug differed between studies.

A critical determinant of the discriminating quality of any test is its reproducibility, i.e., agreement between repeated measurements under base-line conditions. The control group of 12 asthmatics in the Orehek (21) study demonstrated remarkably high reproducibility of PD_{100} for two control measurements done 1 wk apart. Because it is well known that asthma is an unstable disease and the response to bronchial challenge can be modified by various exogenous factors as well, their cohort of asthmatics must have been unusually stable. Although we were not able to attain as narrow a PD_{100} variance as that obtained by Orehek et al. (21), the day-to-day reproducibility of our data, specifically of dose-response curves, showed good agreement with the results reported by other investigators (15, 32). The variability of dose-response curves obtained on our asthmatics is best demonstrated graphically. The left-hand panels of Fig. 4 show the superimposed plots of a set of dose-response curves for two asthmatics, obtained on 7 different days (screening, air-exposure week, NO_2-exposure week). The set of curves with the least variance was obtained in *subj 64*, whereas the largest variability was observed in *subj 69*. To facilitate comparison of postair and post-NO_2 data for the two asthmatics, individual data points, as well as fitted curves, are shown on the right-hand panels. These curves clearly show enhanced post-NO_2 responsiveness, both in terms of attenuated PD_{100} and increased slope.

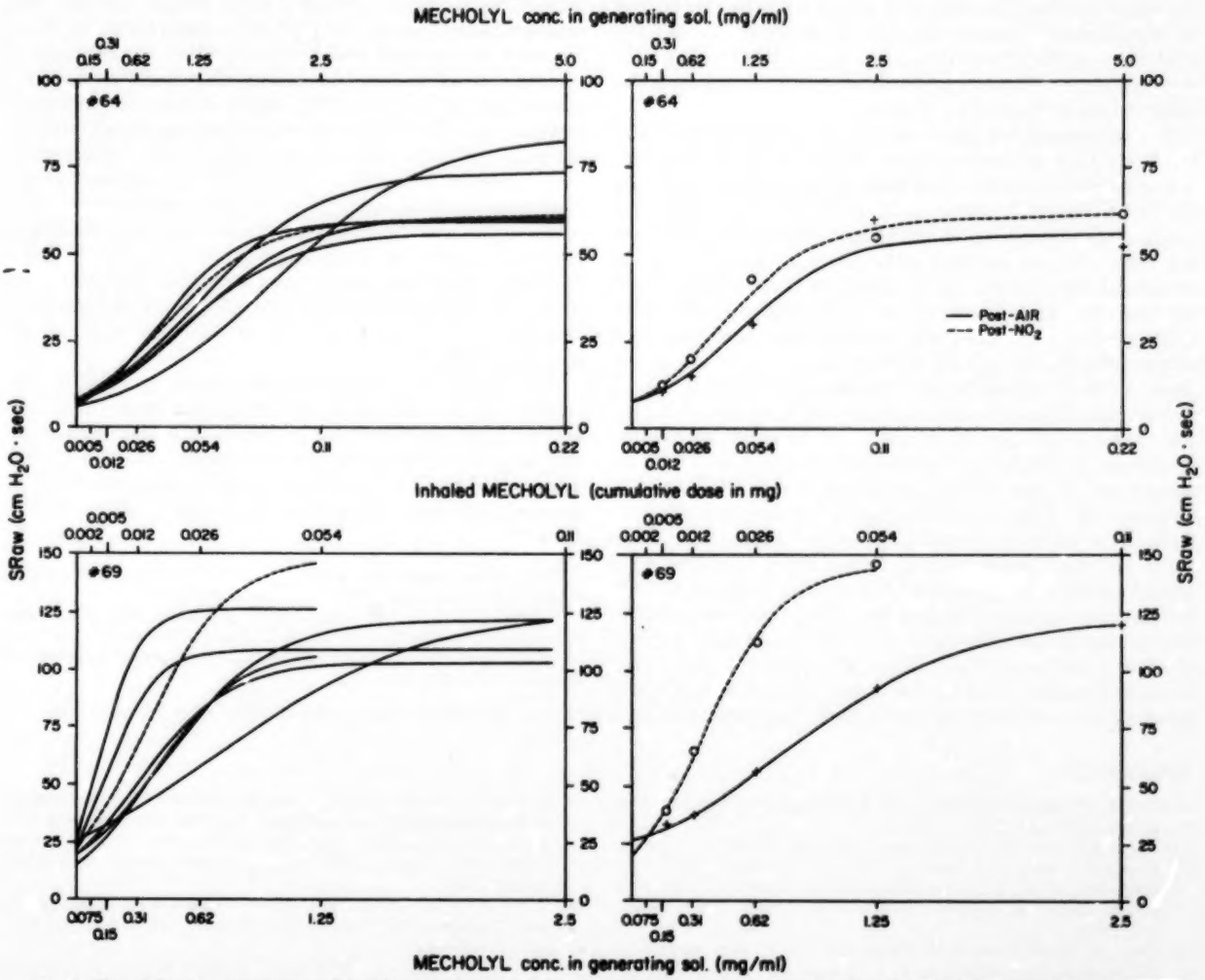

FIG. 4. Plot of dose-response curves for asthmatics *no. 64* and *no. 69* for each of the experimental days. *Subj no. 64* exhibited the least, whereas *subj no. 69* showed the greatest variability of responses. *Broken-line curves* (all *panels*) represent a dose-response obtained after NO_2 exposure. *Left panels* show plots of sRaw vs. methacholine solution concentration-inhaled dose (logistic curves) for each experimental day. *Right panels* show plots of sRaw measured at each methacholine solution concentration-inhaled dose as well as logistic fit over these points (postair = *solid lines*.)

Yet, when these (post-NO_2) changes are compared with data measured during control sessions, the "enhancement" falls within the normal variability range of the subject. Most of our subjects in both groups, normals and asthmatics, had a day-to-day variability of response resembling that of *subj 64* rather than *subj 69*. From these plots, it is apparent that in our study the within-subject variability for bronchoconstrictor dose-response curves, under base-line conditions, is greater than any effect induced by NO_2 exposure (interrupted line).

In selecting the subjects for the study, we used more stringent criteria than Orehek et al. (21). Smokers, women, older subjects, and intrinsic asthmatics were all considered ineligible for our study, whereas such subjects were allowed to participate in Orehek's study. If our selection criteria had been applied to Orehek's cohort, only 7 of his 20 asthmatic subjects would have qualified for participation. It has been shown, however, that demographic factors play a minimal role in airway reactivity; thus the comparative evaluation of data should not be significantly biased (30, 32). In general, we believe that none of the above-discussed methodological or subject-selection differences should seriously jeopardize the comparison of these two studies.

A comparison between our group of asthmatics and Orehek's (21) cohort revealed that 3 of 15 of our asthmatic subjects can be classified as responders (applying Orehek's criteria), whereas 13 of 20 of the asthmatics studied by Orehek et al. (21) were responders. Because we were able to identify only three asthmatics as responders, we did not try to categorize our patients and analyze the data in terms of responder-nonresponder characteristics. Surprisingly enough, the mean base-line sRaw value(s) for our 15 asthmatics are practically the same as those reported by Orehek et al. (21) for his 13 responders. Thus the pre- and post-NO_2 exposure values for mean base-line sRaw prior to bronchoconstrictor challenge in Orehek's responders were 6.0 and 6.9, respectively, vs. our (asthmatic) group mean of 6.3 and 6.8, respectively. They reported this difference as being statistically significant ($P < 0.05$). Had we applied the same statistical test (paired t test) to our data, the difference would have been significant ($P < 0.05$) as well. Similarly, for the same group of responders, they reported a statistically significant difference ($P < 0.001$) between control (postair) and post-NO_2 PD_{100}. By applying the same Student's paired t test to the data obtained on our three asthmatic responders, we could have demonstrated that NO_2-induced changes almost achieved statistical significance ($0.1 > P > 0.05$). In our sample of asthmatics, PD_{100} would have had to decrease about 40% post-NO_2 exposure before significance would have been reached in a one-tailed test at the 0.05 level of significance. PD_{100} of responders in the study by Orehek (21) decreased by 45% and was significant at the 0.001 level. The difference in power between the two studies can be attributed to the larger within-subject variability of PD_{100} observed in our study.

sRaw in our asthmatics would have had to increase about 12% post-NO_2 exposure before significance would have been reached in a one-tailed test at the 0.05 level of significance. Orehek (21) observed a 15% increase that was significant at the 0.05 level of significance. Therefore, for sRaw the two studies were of about equal power. Our failure to find a significant increase in sRaw was because the increase at post-NO_2 exposure was only about 7% as opposed to the 12% needed for significance at the 0.05 level. Considering the experimental design, the reported nonsignificant postair or post-NO_2 differences in PD_{100} between responders and nonresponders (categorized in retrospect) as well as the nonsignificant differences between postair and pre-NO_2 sRaw within the two subgroups, we believe that the statistical approach used by Orehek et al. (21) was not appropriate (10). There seems to be a strong possibility that if more appropriate statistical tests, such as analysis of variance, had been applied to the data and the experimentwise error rate had been corrected using the Bonferroni method (1, 10), null hypotheses would not have been rejected and, as in our study, there would have been no significant difference in sensitivity and reactivity of responders and nonresponders to NO_2.

The negative findings in our study as well as the relative magnitude of the observed base-line variability for the dose-response curves lead us to conclude that exposure to 0.1 ppm NO_2 for 1 h without exercise had no demonstrable airways effects either in young atopic asthmatics with mild disease or in young healthy normal subjects.

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